Reprinted from **Rickettsiology: Current Issues and Perspectives**
Volume 590 of the *Annals of the New York Academy of Sciences*
June 26, 1990

Characterization of *Coxiella burnetii pyrB*

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Aspartate transcarbamoylase (ATCase, EC 2.1.3.2) is a key regulatory enzyme which catalyzes the conversion of L-aspartate and carbamoyl phosphate into carbamoyl-L-aspartate and phosphate. This first committed step in *de novo* pyrimidine biosynthesis channels a common carbamoyl phosphate pool of the pyrimidine and arginine pathways for the synthesis of pyrimidine nucleotides. The pyrimidines uridine triphosphate (UTP) and cytidine triphosphate (CTP), substrates in nucleic acid biosynthesis and a number of other cellular metabolic processes, are end products of the pathway.

The structure and function of ATCase has been well studied in *Escherichia coli*. The regulation of enzyme activity is controlled positively by ATP and negatively by CTP.[1-3] The enzyme exhibits positive cooperativity for the binding of either aspartate or carbamoyl phosphate, displaying a sigmoidal response of reaction velocity to substrate concentration.

ATCase from *E. coli* is a heteromultimer comprised of six catalytic (C) and six regulatory (R) polypeptides, assembled in a $2C_3$-$3R_2$ arrangement.[4-10] The holoenzyme has a molecular mass of 306 kilodaltons (kDa), the catalytic polypeptide being 34 kDa and the regulatory polypeptide 17 kDa. Subunits can be dissociated by heat or mercurial treatment and reassociated with full activity. Catalytic trimers that have been dissociated from regulatory dimers are active but obey Michaelis–Menten kinetics, i.e., do not show cooperativity on substrate binding and are not responsive to nucleotide effectors. Regulatory dimers bind nucleotide effectors but are catalytically inactive. The three-dimensional X-ray crystallographic structure has been resolved to 2.5 Å, and the location and identity of most residues involved in the active and allosteric effector binding sites have been determined.[10-15]

In addition to control at the level of enzyme activity, flux through the pyrimidine pathway in *E. coli* is also controlled at this step at the level of gene expression. The catalytic and regulatory polypeptides are encoded by the bicistronic *pyrBI* operon.[16,17] An attenuation mechanism allows transcription through *pyrBI* only under conditions of limiting pyrimidines. When CTP and UTP pools are low, RNA polymerase proceeds more slowly through a cluster of pyrimidine bases in the message prior to a terminator in a leader gene, allowing translation to couple with transcription. This disrupts the terminator, and the *pyrBI* genes are transcribed. Conversely, when pyrimidines are abundant and RNA polymerase can proceed through the leader rapidly, termination occurs and prevents transcription of *pyrBI*.[16,18-21] The regulatory scheme for the pyrimidine pathway in *E. coli* is

thus multi-faceted, making the formation of carbamoyl aspartate quite sensitive to the levels of substrates and end products.

The *pyrB* gene from *Bacillus subtilis* has also been cloned and sequenced.[22] The *B. subtilis* ATCase is a trimer with 304 amino acids per chain, with no evidence to date for *pyrI*-encoded regulatory polypeptides in this microorganism. The enzyme is unaffected by nucleotides and shows no cooperativity for substrate binding, obeying Michaelis–Menten kinetics. Expression of the gene is controlled by pyrimidine concentration, possibly via attenuation, and by factors responsive to the developmental cycle, i.e., factors which halt synthesis of the *pyrB* gene product at the onset of sporulation.[23,24]

Early studies with the pyrimidine pathway in *C. burnetii* were mainly concerned with the demonstration of various host-independent enzymatic activities. Mallavia and Paretsky[25] showed aspartate transcarbamoylase and ornithine transcarbamoylase activity in extracts from purified organisms. [C^{14}]aspartate and carbamoyl phosphate were converted into carbamoyl-[C^{14}]aspartate, and ornithine and carbamoyl phosphate were converted into citrulline by using crude *C. burnetii* extracts. Our interest in the ATCase of *C. burnetii* centers around the possibility that this enzyme is regulated developmentally during sporulation. This report characterizes the *C. burnetii* gene encoding the aspartate transcarbamoylase polypeptide.

CLONING OF THE *pyrB* GENE

C. burnetii genomic DNA was digested to completion with *Eco*R I, *Hin*d III, *Pst* I, and *Bam*H I. Fragments were ligated into appropriately digested pEMBL8+[26] and transformed into DH5aF′, a male *E. coli* strain. Original selec-

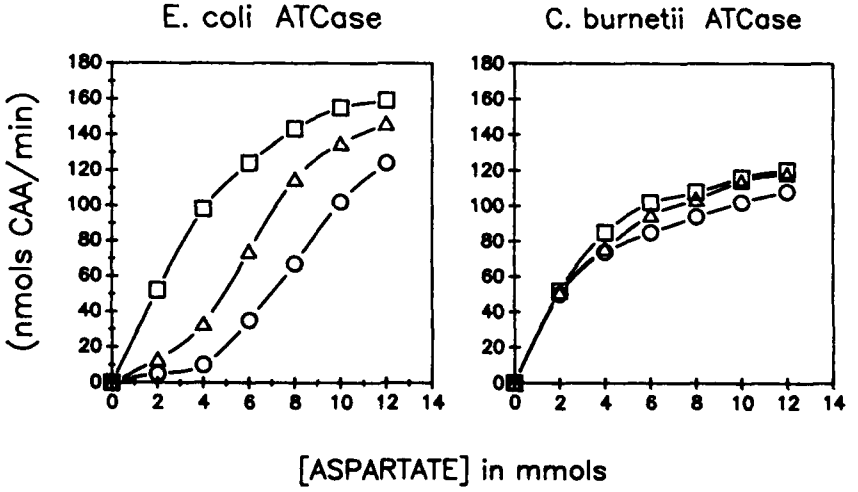

FIGURE 1. *E. coli* and *C. burnetii* ATCase activity from cloned genes. Plots of reaction velocities vs. substrate concentration with extracts from *C. burnetii* and *E. coli pyrB* clones in TB2. *Triangles* represent assays with no effectors present. *Circles* and *squares* represent the addition of 2 m*M* CTP or 2 m*M* ATP, respectively.

tion was made on LB-ampicillin plates. In addition to screening transformants for *C. burnetii* antigens, aliquots of pooled clones were induced to package the plasmids ("phasmids") into phage particles. These phage were then used to infect TB2, an *E. coli* strain with *pyrB* and *argI* deleted. Transfectants were plated onto minimal media supplemented with arginine and ampicillin. Plasmid DNA was purified from 12 independent isolates. Restriction digests with several different enzymes revealed consistent patterns among the 12 clones. This suggested that a single region of the genome had been cloned and that, at least at the level of restriction mapping, there were no gross deletions or rearrangements due to the selective pressure of requiring growth on minimal media.

Enzyme activity from crude, cell-free extracts of the cloned *C. burnetii* ATCase showed a hyperbolic response by reaction velocity to aspartate concentration (FIG. 1). CTP and ATP had little effect on activity. The K_m approximated that of *E. coli* catalytic trimers. The passing of extracts through gel filtration sizing columns or HPLC columns failed to reveal a molecular weight for the enzyme, probably due to the low level of enzyme synthesized. Extracts applied to a 4–20% native polyacrylamide gradient gel, which was electrophoresed for 20 h at 100 volts and stained for enzyme activity, showed bands which migrated more slowly than the catalytic subunit (100 kDa) and faster than the holoenzyme (310 kDa) of *E. coli* (data not shown).

CHARACTERIZATION OF *C. burnetii pyrB*

The insert carrying the putative *pyrB* gene was a 7-kilobase-pair (kbp) *Eco*R I fragment. Southern blot transfer verified that it was *C. burnetii* genomic DNA. Partial *Sau*3A digests of the insert resulted in the subcloning of a 2.2-kbp fragment with the same activity as the parent clone. This fragment was sequenced and a 954-bp open reading frame (ORF) with some homology to the *pyrB* gene of *E. coli* was found.

A purine-rich region 8 bp upstream from the first methionine codon appears to be the site of translation initiation.[27] The deduced amino acid sequence of this ORF would result in a 310-residue polypeptide, compared to a 311 amino acid catalytic polypeptide for *E. coli* and 305 residues for *B. subtilis*, counting the respective initiation methionines. Homology at the nucleotide level between *C. burnetii pyrB* and *E. coli pyrB* was 44%, and between the genes from *C. burnetii* and *B. subtilis* was 50%. An *E. coli*–like promoter was not apparent in the upstream sequence, nor were there any structures resembling an attenuator.[28] An ORF extended from at least 500 bp upstream to the second codon of *pyrB* (FIG. 2). *C. burnetii pyrB* may therefore be part of an operon. The *pyr* genes in *E. coli* were scattered around the chromosome,[29,30] but those in *B. subtilis* were tightly linked, with *pyrA–F* all within a single 10-kbp segment of the chromosome.[31] The upstream ORF sequence was used to search in databases for other similar genes but none was found. A more rigorous comparison was performed between the *E. coli pyrC* (known to be adjacent to *pyrB* in *B. subtilis*) and *pyrI* genes, with no significant regions of similarity observed.

Amino acid homology between catalytic polypeptides, as expected, was higher, with 61% similarity (identical residues and conservative substitutions) between *C. burnetii* and *B. subtilis* ATCase and 57% between *C. burnetii* and *E. coli*. Homology was greater among the three enzymes around residues known to be involved in catalysis. There were important differences for the three en-

zymes in the sequences in regions involved in the interfaces between catalytic and regulatory subunits in the *E. coli* enzyme. The *C. burnetii* and *B. subtilis* enzymes both had deletions (7 and 10 residues, respectively) around residue 210 of the *E. coli* sequence, a region of contacts between subunits in the *E. coli* enzyme. This suggests that the *C. burnetii* ATCase does not bind a regulatory polypeptide, as is the case for *B. subtilis*.

A further indication that *C. burnetii* lacks a regulatory polypeptide was the lack of a *pyrI* gene in an operon with *pyrB*. There were 15 base pairs separating the two genes in *E. coli*.[32,33] There was not an ORF within the 200 base pairs downstream of *C. burnetii pyrB*. There was in fact an unusually strong rho-independent, terminator-like structure 10 base pairs past the translational stop codon.[34] This structure consisted of 34 bases in the stem with just one mismatch (none was a G-U pair) and an 11-base loop. The free energy value was in the −70-kcal range.[35,36] A *pyrI* could, of course, lie elsewhere on the chromosome. In fact, one explanation for the increase in product formation upon the heating of *C. burnetii* extracts in the assays of Mallavia and Paretsky[25] is the dissociation of a regulatory subunit. Our initial attempts to assay the enzyme in disrupted *C.*

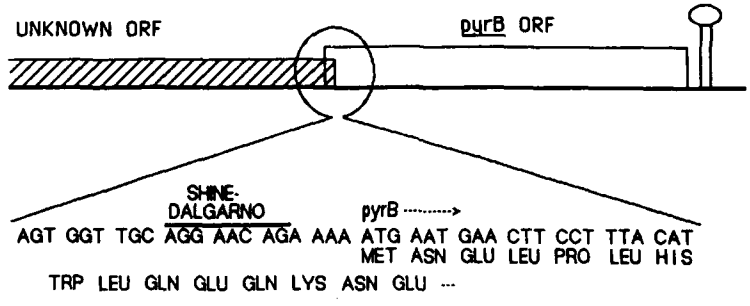

FIGURE 2. Diagram of the *pyrB* locus in *C. burnetii*. The structure following the *pyrB* ORF represents the terminator. The two major ORFs overlap by 5 bp.

burnetii yielded activity too low to answer adequately the questions of substrate cooperativity and feedback inhibition. Successful assays of extracts and the probing of the genome with *pyrI* probes may demonstrate the presence of the gene or provide further evidence that *C. burnetii* lacks it.

SUMMARY

The *C. burnetii pyrB* gene was cloned on a 7-kbp *Eco*R I fragment. DNA sequence analysis, enzyme assays, and amino acid homologies with *E. coli* and *B. subtilis pyrB* gene products suggest that (i) *C. burnetii* ATCase exists as a trimer, (ii) the microorganism may not synthesize a regulatory polypeptide, and (iii) *pyrB* may be part of an operon whose expression is under the control of an upstream promoter. The high degree of homology of the active site further suggests that a common mechanism of catalysis for ATCase exists between such diverse organisms as *C. burnetii*, *E. coli*, and *B. subtilis*.

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